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[28] Enzyme Immunoassay ELISA and EMIT

By EVA ENGVALL

Enzyme immunoassays today can be classified into two fundamentally different types of assays. For want of better nomenclature, these are at present referred to as heterogeneous and homogeneous enzyme immunoassays (EIA).

The heterogeneous enzyme immunoassays, which include the enzyme-linked immunosorbent assay (ELISA),[1-3] are based on the same principles as are used in radioimmunoassays (RIA). In short, after incubation of antigen and antibodies, the antigen–antibody complexes formed are separated from free antigen and antibody by one of a number of different techniques, and the activity in one or both of the fractions is determined.

In the homogeneous enzyme immunoassay[4] (EMIT = enzyme multiplied immunoassay technique), no such separation is necessary. The principle of EMIT is similar to the modified bacteriophage technique.[5] Antigen-coupled enzyme (or bacteriophage) will show a change in activity (infectivity) upon incubation with antibody. This change is inhibited when the binding of antibody to the antigen-coupled enzyme (bacteriophage) is prevented by addition of free antigen.

ELISA is generally applicable to the measurement of almost any antigen. The usefulness of EMIT will probably remain limited to assay of low molecular weight haptens.

In ELISA, the enzyme is a passive passenger through the actual immunoassay. In EMIT, the enzyme plays a key role throughout the assay process.

ELISA requires very little knowledge of enzyme technology, whereas enzymology is the key to success in EMIT.

[1] E. Engvall and P. Perlmann, *Immunochemistry* 8, 871 (1971).
[2] E. Engvall, K. Jonsson, and P. Perlmann, *Biochim. Biophys. Acta* 251, 427 (1971).
[3] E. Engvall and P. Perlmann, *J. Immunol.* 109, 129 (1972).
[4] K. E. Rubenstein, R. S. Schneider, and E. F. Ullman, *Biochem. Biophys. Res. Commun.* 47, 846 (1972).
[5] J. Haimovich, E. Hurwitz, N. Novik, and M. Sela, *Biochim. Biophys. Acta* 207, 125 (1970).

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Classification of EIA Techniques

A brief description of techniques used in EIA follows. The various assays have been classified as either competitive or noncompetitive assays, depending on whether or not the technique involves a reaction step in which unlabeled and labeled antigen compete for a limited number of antibody sites (competitive assay) or whether the antigen (or antibody) to be measured is first allowed to react with antibody (antigen) on a solid phase followed by measurement of the binding of enzyme-labeled immune reactant (noncompetitive assay)

Competitive Assays

ELISA Using Antigen–Enzyme Conjugate. Assays for IgG[5] and HCG,[6] respectively, were the first two examples of quantitative enzyme immunoassay. Both were based on competition of enzyme-labeled antigen with antigen from either standard or unknown sample for the binding to a limited amount of antibody coupled to a solid phase. Figure 1 illustrates the features of the assay. In this type of assay, the first operation is the physical or chemical attachment of an appropriate amount of antibody to a solid phase. This is then incubated with a solution containing a fixed concentration of enzyme-labeled antigen, and no unlabeled antigen, a known but variable concentration of standard antigen, or an unknown concentration of test antigen from a sample is included. The reaction mixture is then incubated until the antigen–antibody reaction attains equilibrium. After washing, the enzyme activity on the solid phase is determined, usually by incubation with substrate buffer for a certain time period. The product concentrations are inversely proportional to the concentrations of standard or test antigen added.

EMIT Using Hapten–Enzyme Conjugate. In this assay, a fixed amount of hapten–enzyme conjugate is incubated with a fixed amount of antibody to the hapten together with a variable amount of free hapten.[7] The antibody will cause a change in enzymic activity upon reaction with one or more haptens on the enzyme. In this assay, equilibrium does not necessary have to be attained before enzyme measurement, no separation of free from antibody-bound hapten is required, and the rate of enzyme reaction is measured, rather than an end point.

ELISA Using Enzyme-Labeled Antibody. Another type of competitive ELISA employs enzyme-labeled antibody with the antigen attached to a solid phase. In this technique, the binding of enzyme-labeled antibody to

[6] B. van Weemen and A. H. W. M. Schuurs, *FEBS Lett.* **15**, 232 (1971).
[7] K. E. Rubenstein, *Scand. J. Immunol.* **8**, Suppl. **7**, 57 (1978).

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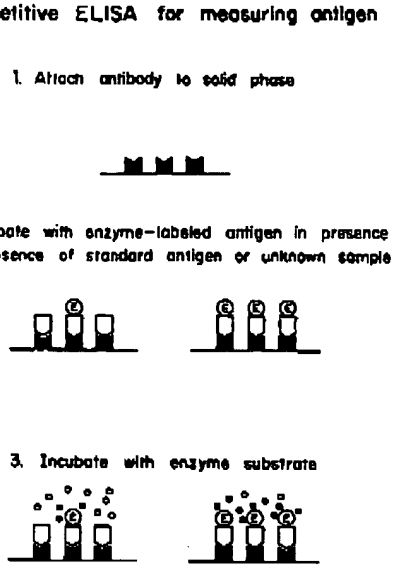

FIG. 1. Scheme for a competitive solid phase enzyme immunoassay. From Engvall and Ruoslahti.[33] Reproduced with permission.

immobilized antigen is competitively inhibited by an added standard or test antigen. As is the case in the competitive ELISA with enzyme-labeled antigen, the product concentrations measured at the end are inversely proportional to the concentrations of the standard or test antigen in the incubation solutions.

In its most versatile and convenient form, this assay is performed as a two-step assay with enzyme-labeled anti-immunoglobulin.[8] Antigen on the solid phase is incubated with a high dilution of antiserum with or without addition of standard antigen or unknown samples. After washing, the antibodies bound to the solid phase are detected using enzyme-labeled anti-immunoglobulin. Semipurified antigen works satisfactorily in this assay, and the antiserum can be of a fairly low titer. The enzyme-labeled anti-immunoglobulin can be readily prepared using conventional procedures. The same conjugate can be used for the assay of several different antigens, provided that the primary antisera have been prepared in the same species.

[8] E. Engvall and P. Perlmann, *in* "Automation in Microbiology and Immunology" (C.-G. Heden and T. Illeni, eds.), p. 529. Wiley, New York, 1975.

Noncompetitive Assays

"Sandwich" Assay. Immobilized antibody in excess is incubated with standard or test antigen (Fig. 2). After washing, the immobilized antibody–antigen complex is incubated with an excess of enzyme-labeled antibody which binds to one or more remaining antigenic sites.[9] Alternatively, the second antibody may be unlabeled, and the procedure is expanded to include an incubation with excess enzyme-labeled third antibody specific for IgG of the animal species in which the second antibody is elicited.[10] In the latter case, the immobilized and second antibodies must be obtained from different animal species in order to prevent the binding of enzyme-labeled third antibody directly to the immobilized antibody. In both variants, the concentration of the product from the enzyme reaction is directly proportional to the concentration of standard or test antigen.

Assays for Measuring Antibody. Another type of noncompetitive

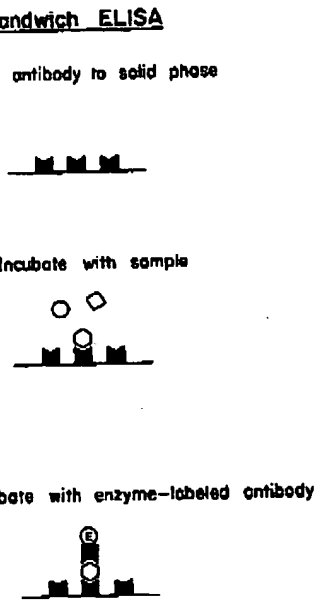

FIG. 2. Scheme for a noncompetitive solid phase enzyme immunoassay. From Engvall and Ruoslahti.[22] Reproduced with permission.

[9] L. Belanger, C. Sylvestre, and D. Dufour, *Clin. Chim. Acta* **48**, 15 (1973).
[10] A. R. Frackelton, Jr., R. P. Szaro, and J. K. Weltman, *Cancer Res.* **36**, 2845 (1976).

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. 36, 2845 (1976).

ELISA is the indirect method for measuring antibody concentration. This procedure employs immobilized antigen and enzyme-labeled second antibody against IgG of the species in which the test antibody has been elicited. This method has been used to measure antibodies to a variety of antigens.[3,11,12]

Factors Involved in the Choice of Assay Design

Although competitive ELISA techniques are specific and easy to execute, they also suffer from several disadvantages. To perform a competitive ELISA using enzyme-labeled antigen, purified antigen in relatively large amounts is required for preparation of the enzyme–antigen conjugate. In cases where purified antigen is not available, a variant of the competitive ELISA method employing enzyme-labeled antibody or anti-immunoglobulin can be used.

A more serious problem in the application of the competitive ELISA relates to the difficulties caused by the need to incubate enzyme-labeled antigens or antibodies with biological fluids such as serum, urine, or tissue extract. These fluids contain proteases, and noncompetitive enzyme inhibitors may also be present. Such substances, when present, may alter the activity of the enzyme used as label. This difficulty is avoided in the noncompetitive ELISA techniques in which the incubation with test samples is carried out separately from the incubation with enzyme-labeled antigen or antibody. The noncompetitive ELISA offers additional advantages. Since most of such assays employ enzyme-labeled antibodies, the purification and specific enzyme-labeling of individual antigens is not necessary. Thus, the same enzyme-labeling procedure and solid phase attachment method can be used for different antibodies. Another advantage of the noncompetitive ELISA is the possibility of binding several enzyme-labeled antibody molecules to a single polyvalent antigen molecule, thus providing an element of amplification. This may be an advantage in procedures in which the ultimate sensitivity has not been attained, i.e., the sensitivity limit is not set by the affinity between the antigen and antibody.

Preparation of Enzyme Conjugate

Selection of Enzyme for ELISA. In the following, some characteristics of an enzyme suitable for enzyme immunoassay are discussed and attention is focused on the characteristics of enzyme that will influence the sensitivity, practicality, and cost of the assays.

[11] H. E. Carlsson and A. A. Lindberg, *Scand. J. Immunol.* **8**, Suppl. 7, 97 (1978).
[12] A. Voller, A. Bartlett, and D. E. Bidwell, *Scand. J. Immunol.* **8**, Suppl. 7, 125 (1978).

First, the enzyme should be conveniently detectable at or below the nanogram level. This not only means that the enzyme should have a high specific activity, i.e., convert a high number of substrate molecules into product molecules per time unit, but also requires that the product be detectable with high sensitivity.

An important consideration in the choice of enzyme is also that the samples to be measured should not contain substances that could interfere with the activity of the enzyme or its measurement. This applies primarily but not exclusively to assays in which the enzyme-labeled compound is incubated together with the sample. Some obvious examples include the need to avoid samples containing EDTA if the enzyme utilized requires metals for activity. Similarly, oxidoreductases cannot be used when the samples contain some commonly used preservatives. Endogenous enzyme inhibitors or other substances interfering with the activity or stability of the enzymes may also be present in the samples to be assayed.

Endogenous enzymic activity will probably not interfere in a solid phase enzyme immunoassay where the activity bound to the solid phase is measured. It is evident that endogenous enzymes can interfere in EMIT-type assays.

Other trivial but nonetheless important aspects in the choice of enzymes include availability, cost, and shelf life.

Choice of Enzyme for EMIT. Since EMIT does not include any separation of immune reactants from sample before enzyme assay, the choice of enzyme becomes restricted to a much less variable group than those that can be used for ELISA.

It is of prime importance in EMIT that an enzyme be chosen that is not present in the test sample. Furthermore, the test sample must not contain inhibitors of the enzyme chosen, nor appreciable amounts of enzyme substrate. In addition, the enzyme must change its catalytic properties in a measurable way after interaction with antibodies.

Most enzymes are not inhibited by antibodies. However, enzymes acting on high molecular weight substrates, can be sterically hindered in their activity by antibodies. This is the basis for the use of lysozyme in homogeneous enzyme immunoassays.[4] The situation is more complex in the case of two other enzymes, malate dehydrogenase and glucose-6-phosphate dehydrogenase, which are also extensively used in EMIT-type assays. Substitution of unique residues in the molecule with hapten apparently makes the enzyme susceptible to conformational changes upon reaction with antibody, and this leads to inhibition of enzyme activity.[18]

[13] G. L. Rowley, K. E. Rubenstein, J. Huisjen, and E. F. Ullman, *J. Biol. Chem.* 250, 3759 (1975).

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Biol. Chem. 250, 3759

REAGENTS FOR COUPLING ENZYMES TO PROTEINS

Compound	Reacting group in protein	Reference
Glutaraldehyde	—NH_2	*a, b*
Toluene diisocyanate	—NH_2	*c, d*
p,p'-Difluoro-*m,m'*-dinitrophenyl sulfone	—NH_2	*e*
Carbodiimides	—COOH —NH_2	*f, g*
p-Benzoquinone	—NH_2 —SH	*h*
N,N'-o-Phenylenedimaleimide	—SH	*i*
m-Periodate	—NH_2 —CHOH	*j, k*

[a] S. Avrameas, *Immunochemistry* 6, 43 (1969).
[b] S. Avrameas and T. Ternynck, *Immunochemistry* 8, 1175 (1971).
[c] A. F. Schick and S. J. Singer, *J. Biol. Chem.* 236, 2477 (1961).
[d] R. R. Modesto and A. J. Pesce, *Biochim. Biophys. Acta* 229, 384 (1971).
[e] S. S. Tawde and J. S. Ram, *Arch. Biochem. Biophys.* 97, 429 (1962).
[f] S. Avrameas and J. Uriel, *C. R. Acad. Sci.* 262, 2543 (1966).
[g] P. K. Nakane, J. S. Ram, and G. B. Pierce, *J. Histochem. Cytochem.* 14, 789 (1966).
[h] T. Ternynck and S. Avrameas, *Immunochemistry* 14, 767 (1977).
[i] K. Kato, Y. Hamaguchi, H. Fukui, and E. Ishikawa, *J. Biochem.* 78, 235 (1975); *Eur. J. Biochem.* 62, 285 (1976).
[j] P. K. Nakane and A. Kawaoi, *J. Histochem. Cytochem.* 22, 1084 (1974).
[k] A. Murayama, K. Shimada, and T. Yamamoto, *Immunochemistry* 15, 523 (1978).

An interesting exception to this generalization is the increased activity exhibited by malate dehydrogenase which has been coupled to thyroxine, in the presence of antibodies to thyroxine.[7]

Labeling of Antigens and Antibodies with Enzyme. Many methods exist for coupling haptens, proteins, and carbohydrates to proteins. Some of the most commonly used are summarized in the table together with some selected references. An extensive review on protein–protein coupling reactions has recently appeared[14] and could be used as a source of additional chemical details on the coupling methods.

Reagents for protein–protein coupling are generally nonspecific in that they react with functional groups that are common to all proteins. The extent of intermolecular as opposed to intramolecular cross-linking will depend on the relative number and availability of such functional

[14] J. H. Kennedy, L. J. Kricka, and P. Wilding, *Clin. Chim. Acta* 70, 1 (1976).

groups in the two proteins to be conjugated. The number of total functional groups present in any given protein can be determined, but whether these are available for intermolecular cross-linking to another protein is not so easily tested. The optimal conditions for conjugating two proteins to each other will, therefore, have to be determined by trial and error.

Glutaraldehyde as a Cross-Linking Agent. Glutaraldehyde (GDA) is the cross-linking agent used most extensively in enzyme immunoassay.[15] It is a dialdehyde, and theoretically could cross-link two proteins via the ε-amino groups of lysine by formation of a Schiff's base. However, the observed stability of proteins cross-linked with GDA does not agree with the known reversibility of Schiff's base formation. The mechanism for GDA cross-linking involving polymerization products of GDA has been proposed by Richards and Knowles,[16] and agrees better with the efficiency of coupling and the stability of the resulting conjugates. We have always used technical grade GDA, which is presumably extensively polymerized, for conjugations and have obtained good and reproducible results. Others have reported good results with the use of GDA purified by distillation. However, I have talked to several investigators who have been unable to obtain cross-linking in spite of adherence to published procedures, and it has, without exception, become evident that they have been using highly purified GDA.

Cross-Linking via Carbohydrate Moiety by Using Periodate. An interesting and potentially very useful method for coupling peroxidase to antigens and antibodies in EIA was introduced by Nakane and Kawaoi.[17] The carbohydrate moiety of horseradish peroxidase was oxidized with sodium periodate, and the resulting aldehyde groups were allowed to react with amino groups of antibody. The reverse approach was explored by conjugating peroxidase and lysozyme to periodate-oxidized antibody.[18] The advantage of utilizing the carbohydrate moiety of antibodies and enzymes is obvious, considering that carbohydrate is not essential for either the immunological activity of antibodies or the catalytic property of enzymes.

Characteristics of Enzyme Conjugates. Most conjugation procedures give rise to products heterogeneous in size and composition. After conjugation of antigen (or antibody) to an enzyme, the reaction mixture may thus be composed of free antigen (antibody), free enzyme, and one or more antigen (antibody) molecules linked to one or more enzyme molecules.

The presence of free antigen (or antibody) will lower the specific activ-

[15] S. Avrameas, *Immunochemistry* 6, 43 (1969).
[16] F. M. Richards and J. R. Knowles, *J. Mol. Biol.* 37, 231 (1968).
[17] P. K. Nakane and A. Kawaoi, *J. Histochim. Cytochem.* 22, 1084 (1974).
[18] A. Murayama, K. Shimada, and T. Yamamoto, *Immunochemistry* 15, 523 (1978).

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ity of the conjugate and should be minimized by design of the coupling procedure and by purification of the conjugate. For instance, if the enzyme itself or the conjugate are of higher molecular weight than the antigen, elimination of unconjugated antigen can be accomplished fairly easily by gel filtration on an appropriate matrix. Another way, which to my knowledge has not yet been tried, would be by affinity chromatography or immunochromatography specific for the enzyme.

Free enzyme in the conjugate will interfere mainly in the methods where the activity of the "free" fraction is measured. In most cases, however, the activity is measured in the "bound" fraction, and the presence of free enzyme will cause only minor interference in the form of increased background. If the enzyme is smaller than the labeled compound, free enzyme can be eliminated by gel filtration as above. Immunoadsorbent purification could be useful in separating enzyme-labeled (and free) antigen (antibody) from free enzyme, provided that an elution buffer can be found that does not inactivate the enzyme label.

As already mentioned, most conjugation methods in use give rise to high molecular weight complexes, each containing several enzyme molecules bound to several antigen molecules. In a solid phase assay, only one of the antigen molecules in such a complex will be capable of reacting with an antibody. Since this antigen in the conjugate is associated with several enzyme molecules, the conjugate will exhibit a high specific activity. On the other hand, since only one out of several antigens will express immunoreactivity at a given time, the yield of antigen after conjugation will seem low.

The different nature of the large enzyme–antigen complexes compared to standard antigen does not seem to present any problems in EIA. As in competitive RIA, results from competitive EIA are always obtained by comparing the unknown sample with that of an unlabeled standard antigen. Similarly, in an antibody assay, the results should preferably be compared to a standard antiserum and not expressed as radioactivity or enzyme activity, respectively, which will most likely vary from one laboratory to another, even if the same labeled preparation is used.

Immobilization of Antigen or Antibody. The characteristic that distinguishes ELISA from other EIA is the use of an immune adsorbent to effect a rapid, facile separation of "free" antigen and antibody from antigen–antibody complexes. Methods where antibody and antigen are covalently attached to cellulose, agarose, or polyacrylamide have been described. With the exception of the case where such particles have been made magnetic[19] and can be separated in a magnetic field, the use of par-

[19] J.-L. Guesdon and S. Avrameas, *Immunochemistry* 14, 443 (1977).

ticulate solid phases entails centrifugation in the washing and separation steps. Solid phase carriers, such as large beads, disks, and tubes, facilitate washing and separation steps. Thus, macromolecular antigens and antibodies have been physically adsorbed to plastic carriers (polystyrene, polyvinyl, polypropylene, polycarbonate), and to silicone rubber or treated glass. Indeed, part of the success of ELISA methods arises from the use of disposable polystyrene Microtiter plates[12] or tubes[2] as the solid phase carriers.

Most proteins adsorb to plastic surfaces, probably as a result of hydrophobic interactions between nonpolar protein substructures and the nonpolar plastic matrix. The rate and extent of coating will depend on the diffusion coefficient of the adsorbing molecule, the ratio of the surface area to be coated to the volume of coating solution, the concentration of the adsorbing substances, the temperature, and the duration of the adsorption reaction. Clear polystyrene has been the most widely used support in ELISA methods, and it can be coated easily and reproducibly. However, there are shortcomings in using polystyrene or any other plastic as solid phase. One difficulty is due to the fact that the antigen or antibody is only physically adsorbed, not covalently bound to the solid phase. This type of an immune adsorbent "bleeds" (i.e., loses some of the adsorbed protein during washes and incubations). Furthermore, adsorbed proteins may also undergo denaturation to some extent with loss of immunological activity. The loss of adsorbed antigen or antibody, which amounts to approximately 30% for the time of an assay, lowers the precision of the assay and probably also affects its sensitivity, especially in competitive ELISA techniques. Another disadvantage is that plastic surfaces have a limited capacity of adsorption. However, the ease and rapidity of separation of antigen–antibody complex from "free" antigen and antibody mostly compensates for these drawbacks.

The adsorption process, unlike antigen–antibody interactions, is nonspecific. Thus, during the incubation of the immobilized antigen or antibody with enzyme-labeled antigen or antibody, the latter binds specifically to the immobilized immune reactant, but may also be adsorbed directly onto the solid phase. This nonspecific adsorption of enzyme activity can be minimized by inclusion of a nonionic detergent such as Triton X-100 or Tween 20. These do not interfere with the antigen–antibody reaction but prevent formation of new hydrophobic interactions between added proteins and the solid phase without disrupting to any appreciable extent the hydrophobic bonds already formed between the previously adsorbed protein and the plastic surface.

The optimal concentration of antigen or antibody for coating is generally between 1 and 10 μg/ml. Higher concentrations lead to increased

adsorption, but the percentage adsorbed becomes less. Furthermore, high concentration of protein during coating leads to increased desorption during incubations with immunoreactants. This sometimes gives rise to undesirable prozone phenomena.

Enzyme Affinity Assay. The ELISA type assays are also applicable to studies of nonimmunological interactions. Examples of potentially useful applications include assays of various hormone receptors, where radiochemical techniques are currently used to measure the few receptors for which assays are available. To illustrate the possibilities offered by tests that might be called enzyme affinity assays, I will briefly mention here our recent studies on the utilization of such assays to study the interaction of fibronectin with collagen.[20,21] Fibronectin is a cell surface protein also present in the circulation. It has affinity to collagen, and evidence from *in vitro* experiments suggests that its function on the cell surface is to attach cells to the extracellular collagenous matrix. The circulating form may enhance opsonization of particulate matter containing collagen and fibrin.

Our recent studies on fibronectin have centered around its collagen-binding properties. We originally demonstrated this binding using an

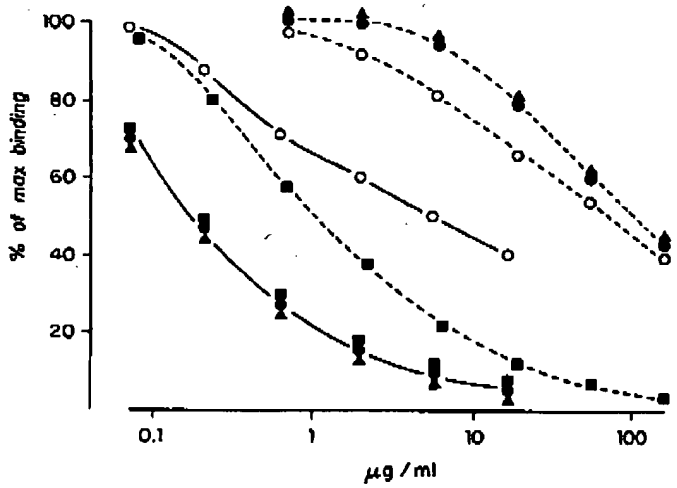

FIG. 3. Inhibition of binding of fibronectin to Microtiter plates coated with gelatin by collagen type I (●), type II (▲), and type III (■) and by AB chains (○). —, Native proteins; ---, heat-denatured proteins. From Engvall *et al.*[21] Reproduced with permission.

[20] E. Engvall and E. Ruoslahti, *Int. J. Cancer* 20, 1 (1977).
[21] E. Engvall, E. Ruoslahti, and E. J. Miller, *J. Exp. Med.* 147, 1584 (1978).

ELISA.[20] We could demonstrate the binding of fibronectin to collagen-coated Microtiter plate wells by detecting the bound fibronectin with enzme-labeled antifibronectin. We have applied this assay to studies on the fibronectin-binding activity of different genetic types of collagens.[21]

Microtiter wells were coated with gelatin (denatured collagen). Fibronectin binds to such wells and can be detected using anti-fibronectin labeled with alkaline phosphatase. Collagenous proteins inhibit the binding of fibronectin to the gelatin-coated wells, and this allows measurement of their relative avidities in binding to fibronectin. Figure 3 shows an example of the results obtained. It could be established that of the native collagens, type III collagen was the most active. Denaturation of the collagens increased their activities. Denatured types I, II, and III collagens were equally active, and the collagen containing the A and B chains remained less active than the other types. More recently, we have modified the assay to a simple affinity assay by attaching the enzyme directly to fibronectin.[22]

ELISA in Practice

Purification of Immunoglobulins and Anti-immunoglobulins. The IgG fraction of an antiserum can be purified according to any of a number of established techniques. If the antiserum was raised in rabbits, the most convenient purification procedure to use is affinity chromatography on protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Excellent yield and purity is obtained by following the recommendations of the manufacturer.

We usually prepare purified antibodies from anti-immunoglobulin sera rather than use the whole IgG fraction. This can be accomplished by a relatively simple procedure, and the use of purified antibodies results in more efficient conjugates than those prepared from whole IgG, and it also saves enzyme.

The procedure we used in isolating antibodies is as follows: An immunoadsorbent can be prepared by coupling IgG (or other immunoglobulin) to Sepharose. Before use, the immunosorbent is washed with the buffer used later for eluting the antibodies followed by washing with phosphate-buffered saline (PBS), pH 7.2. The anti-immunoglobulin serum of the proper specificity (heavy chain-specific or Ig-specific) is inactivated (30 min, 56°) and applied to the column. The column is then washed with PBS until the absorbance at 280 nm is consistently low. The antibodies

[22] E. Engvall and E. Ruoslahti, *in* "Immunoassays in the Clinical Laboratory" (R. M. Nakamura *et al.*, eds.), pp. 89–97. Liss, New York, 1979.

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are then recovered from the column by eluting with 0.1 *M* glycine-HCl buffer at pH 2.6 and collected in fractions. Peak fractions (as measured by absorbance at 280 nm) are pooled, neutralized with solid Tris salt, and dialyzed against PBS or bicarbonate buffer, depending on the purpose (see below). A precipitate will form upon neutralization. This precipitate probably represents antibody complexed with antigen leaking from the column during elution. The precipitate is removed by centrifugation.

Coupling of Antibodies to Horseradish Peroxidase (HRP). We use a slight modification of the two-step glutaraldehyde procedure of Avrameas and Ternynck.[23] The HRP (10 mg, Sigma type VI, RZ = 3) is dissolved in 0.2 ml of 1.25% glutaraldehyde (technical grade), in PBS, pH 7.2, and left at room temperature overnight. The reaction mixture is then diluted to 1 ml and dialyzed against 0.1 *M* carbonate buffer, pH 9.2 (two changes, 1 liter, 4 hr each). IgG or purified antibodies (5 mg) in 0.25 ml of the carbonate buffer is added, and the mixture is incubated again overnight at room temperature. Possible remaining reactive groups are blocked by the addition of 0.1 ml of 0.2 *M* lysine. The conjugate can be stored in 50% glycerol.

The two-step procedure leads to more efficient conjugates than a one-step procedure when peroxidase is used for labeling. The reason is that peroxidase has few free amino groups available for reaction with glutaraldehyde, whereas IgG has many. If HRP and IgG are incubated together with glutaraldehyde, IgG will effectively compete with HRP for available GDA, leading to extensive intermolecular cross-linking of IgG with little coupling to HRP.

Detection of HRP Activity. The substrate for HRP used in assays is H_2O_2 or sometimes other peroxides. The cleavage of H_2O_2 is coupled to the oxidation of a hydrogen donor (chromogen) and goes through several intermediary steps with different rate constants.

There are a variety of chromogens available yielding attractive colors. When choosing a particular color that suits one's taste or matches one's equipment, one should be aware of the fact that recipes for HRP activity measurements in the literature are not always optimal. Other important points to note are that all chromogens are light sensitive and that their colored products tend to stick on surfaces.

The following gives, for the convenience of newcomers in the ELISA field, some colorimetric methods for measuring HRP activity. Some of the methods are taken from the literature, and some are our own modifications of published assays.

Chromogen: 2,2'-Azino-di(3-ethyl-benzthiazoline Sulfonic Acid-6)

[23] S. Avrameas and T. Ternynck, *Immunochemistry* **8**, 1175 (1971).

Ammonium Salt (ABTS). This method is modified from Saunders.[24] The substrate buffer contains 1 mg of ABTS per milliliter and 0.003% H_2O_2 in 0.1 *M* citrate–phosphate buffer, pH 4.0. The enzyme reaction is stopped by addition of ½ volume of 37 m*M* NaCN. The green color is measured at 415 nm.

NaCN effectively inactivates the enzyme without affecting the color, but it is poisonous and smells bad. However, it has been difficult to find a more suitable reagent that would inactivate the enzyme without affecting the color. Lowering the pH by addition of HCl or H_4SO_4 increases the color development nonspecifically, whereas elevating the pH by addition of NaOH or inactivation of the enzyme with NaN_3 similarly reduces the color. In solid phase assays, where the enzyme activity on the solid phase is measured, the best way to stop further color development is to separate the substrate solution from the enzyme.[25]

Chromogen: o-Phenylenediamine. This method, modified from Wolters *et al.*,[26] utilizes 0.4 mg of *o*-phenylenediamine per milliliter and 0.01% H_2O_2 in 0.1 *M* citrate phosphate buffer, pH 5.0. The enzymic reaction is stopped by addition of ½ volume of 4 *N* H_2SO_4. The tangerine colored product is completely soluble but somewhat light sensitive. It is measured at 492 nm.

Chromogen: o-Dianisidine. This method is modified from Avrameas and Guilbert.[27] To 60 ml of 0.1 *M* citrate phosphate buffer, pH 5.0, is added 12 μl of 30% H_2O_2 and 0.5 ml of 10 mg/ml *o*-dianisidine in methanol. The enzymic reaction is stopped by addition of 50 μl of 5 *N* HCl per milliliter of enzyme–substrate mixture. The yellow-orange color is measured at 400 nm. It is more stable than the colored product from *o*-phenylenediamine, but has a tendency to precipitate on solid surfaces.

Other Chromogens Used in EIA. These include 5-aminosalicylic acid,[28] 3,3′-dimethyloxybenzidine,[19] and *p*-cresol,[17] which gives a fluorescent product.

Labeling of Antibodies with Alkaline Phosphatase

Alkaline phosphatase from calf intestinal mucosa (Sigma type VII), a 5 mg/ml suspension in 3.4 *M* $(NH_4)_2SO_4$, 0.3 ml is added to 0.1 ml of a solution of antibody, 5 mg/ml in PBS. If the antibody solution is available

[24] G. S. Saunders, Report at the American Society of Clinical Pathologist Workshop, Chicago, 1977.
[25] R. Maiolini and R. Masseyeff, *J. Immunol. Methods* 8, 223 (1975).
[26] G. Wolters, L. J. Kuijpers, J. Kačaki, and A. Schuurs, *J. Clin. Pathol.* 29, 873 (1976).
[27] S. Avrameas and B. Guilbert, *Biochimie* 54, 837 (1972).
[28] B. van Weemen and A. H. W. M. Schuurs, *FEBS Lett.* 15, 232 (1971).

only in concentrations lower than 5 mg/ml, the enzyme suspension is first centrifuged and the supernatant is replaced by a suitable amount of the antibody solution. The mixture of enzyme and antibody is dialyzed overnight against 1 liter of PBS. Glutaraldehyde in PBS is then added to give a final concentration of 0.2%.[1-3] The conjugation is allowed to proceed for 2–3 hr at room temperature, during this time the initially colorless solution becomes pale yellow. The conjugate can then be diluted to any desired volume and dialyzed free of excess GDA. The conjugate is stored in PBS or Tris buffer, pH 8; an unrelated protein (e.g., serum albumin) is added for stabilization, and a preservative (NaN_3) to prevent microbial growth.

Alkaline phosphatase is highly reactive with GDA and forms cross-linked polymers. However, it is not easily cross-linked to the extent that it will precipitate, as in the case with IgG. Alkaline phosphatase and IgG in a ratio of $\geq 3:1$ (w/w) will form efficient conjugates with negligible amounts of free IgG. The conjugate will not precipitate out of solution even with increasing amounts of GDA.

Alkaline phosphatase can also be coupled to antigens and antibodies using a two-step glutaraldehyde procedure. In the case of coupling to IgG, a two-step procedure does not seem to be of any advantage. However, in the case of labeling of staphylococcal protein A with alkaline phosphatase, a two-step procedure was found to be the only way to obtain workable conjugates,[29] and with fibronectin it was the most efficient way.[22]

Measurement of Alkaline Phosphatase (ALP) Activity. Although a multitude of methods are being used to measure peroxidase, most workers use the following method to measure ALP. The reaction utilizes 1 mg of *p*-nitrophenylphosphate (Sigma) per milliliter in 1 *M* diethanolamine buffer, pH 9.8. The reaction is stopped by addition of ¼ volume of 2 *M* NaOH. The yellow *p*-nitrophenol is measured at 405 nm.

Stepwise ELISA Assay

As one example of how to proceed in setting up ELISA, I have chosen to illustrate a system involving determination of mouse antibodies to human α-fetoprotein (hAFP). I will also show how this assay can be utilized for the quantitative determination of the antigen, AFP. These assays, which we use to characterize monoclonal hybridoma antibodies to AFP,[29a] have been developed with emphasis on simplicity and rapidity rather than sensitivity.

Preparation and Testing of Conjugate. First a conjugate, rabbit anti-

[29] E. Engvall, *Scand. J. Immunol.* **8**, Suppl. 7, 25 (1978).
[29a] M. Uotila, E. Engvall, and E. Ruoslahti, *Mol. Immunol.*, in press.

mouse IgG, is prepared. Purified rabbit antibodies to mouse IgG, 1 mg, are coupled to 3 mg of calf intestinal alkaline phosphatase by a one-step glutaraldehyde procedure as described above. The conjugate is diluted to 2 ml after preparation. The efficiency of the conjugate is tested in microtiter plate wells coated with normal mouse serum in the following way.

Mouse serum, 0.2 ml, diluted 1:10,000 (~1 μg mouse IgG per milliliter) in 0.1 *M* $NaHCO_3$ is added to each of a number of wells in a Microtiter plate. The plate is incubated in a humid chamber for 3 hr at 37°. The plate is then washed twice with 0.05% Tween 20 in 0.9% NaCl. The washing at this and the following occasions is done so that the plate is turned upside down and the contents of the wells are shaken out, and the plate is hit hard on a paper towel. It is then turned right side up and flushed with washing solution from, e.g., a squeeze bottle. Air bubbles must not be trapped in the wells. The plate with all wells filled with wash solution is left for 2–3 min, after which the procedure is repeated.

After the second wash, all wells are filled with 0.2 ml of PBS, pH 7.2, containing 0.05% Tween 20 (incubation buffer). The conjugate is then serially diluted by adding 50 μl to the first well(s), mixing, transferring 50 μl to the next well(s), mixing, etc. (fivefold dilutions). The plate is then incubated in a humid chamber at room temperature overnight. The next morning, the plate is washed three times. The enzyme remaining in individual wells is determined by adding 0.2 ml of the substrate solution (1 mg of *p*-nitrophenylphosphate per milliliter in 1 *M* diethanolamine buffer, pH 9.8) to each well. The plate is incubated at room temperature for 15 min. At this time point, chosen arbitrarily, the enzyme is inactivated by the addition of 50 μl of 2 *M* NaOH.

For absorbance measurement, the content of each well is diluted with 0.75 ml of distilled water and then measured at 405 nm. Figure 4 shows the results.

For further assays, a conjugate dilution of 1:500 was chosen.

Determination of Antibodies to AFP. Wells in a microtiter plate are coated with 1 μg of hAFP. After washing, antisera and normal sera are serially diluted in the wells, and the plate is incubated at room temperature for 3 hr. The plate is then washed three times and 0.2 ml of the conjugate, diluted 1:500 in the incubation buffer, is added to each well. The plate is again incubated for 3 hr at room temperature, washed three times, and incubated with the substrate solution for 15 min. The enzyme is inactivated with NaOH, and the content of each well is diluted in distilled water before measurement at 405 mm. Figure 5 shows the result of titration of a mouse antiserum to hAFP and of a normal mouse serum. There is a direct relationship between the amount of antibodies in the serum and the absorbance measured. This relationship is linear within a certain

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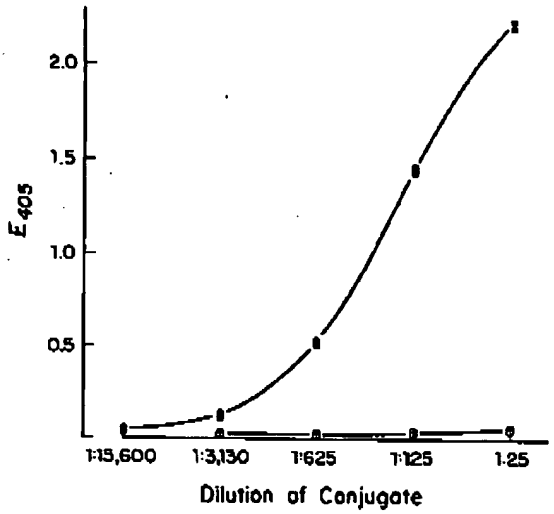

FIG. 4. Testing of a conjugate prepared from purified antibodies to mouse immunoglobulin labeled with alkaline phosphatase. Ordinate: conjugate dilution; abscissa: absorbance at 405 nm of samples diluted 1:4 after 15 min of incubation with substrate. ●—●, Dose response in microtiter wells coated with mouse serum proteins; O—O, dose response in uncoated wells.

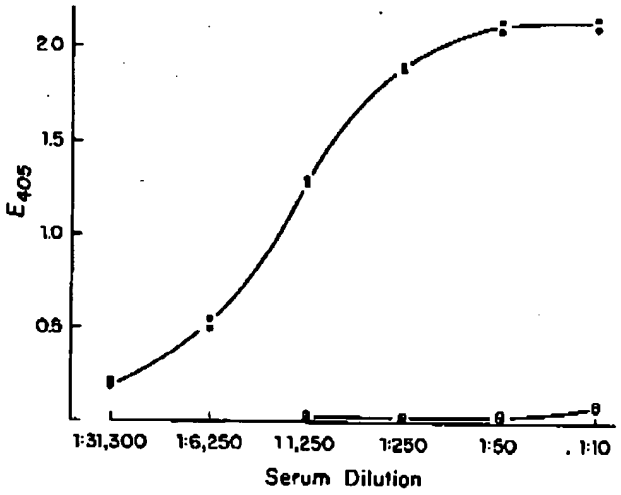

FIG. 5. Titration of mouse anti-α-fetoprotein (AFP) (●) and normal mouse serum (O) in microtiter wells coated with AFP.

range of antibody concentration. At high concentrations of antiserum, the dose response curve levels off because either the amount of antigen on the solid phase or the amount of antibodies in the conjugate becomes a limiting factor.

In general, dose response curves generated from different antisera are parallel. Lack of parallelism can be due to two main effects.

1. The antisera have different specificities. If one serum contains antibodies against a major component of the antigen used for coating, and another contains antibodies against a minor component, the latter is going to give a dose response curve with a plateau level lower than the former.
2. The sera contain antibodies of different immunoglobulin classes, toward which the conjugate reacts with different effectiveness.

A certain "background" is always seen with normal sera in this kind of assay. This background may be due to specific or nonspecific binding of IgG in normal serum to the coated plastic and probably to other unknown protein–protein interactions as well. This normal serum background is usually no problem in experimental animal systems since it varies little from animal to animal. This in turn is probably a consequence of the relative genetic and environmental homogeneity of such species. The "normal" serum background with human sera presents more of a problem because it can be highly variable, and a range has to be established for each type of assay.

Determination of Antigen by Two-Step Competitive Assay Antigen can be quantitated by its capacity to inhibit the binding of antibody to the antigen adsorbed on the solid phase. The affinity of the antiserum is the most important factor among variables that determine the sensitivity of the assay. To obtain maximal sensitivity in the assay, the amount of antigen used for coating is decreased as far as practicable and the amount of antibody added should be limited.

To determine the concentrations of antigen and antibody to be used in a competitive two-step assay, we make checkerboard titrations of the antigen used for coating and of the antiserum. Figure 6A and B shows the result of an experiment in which various concentrations of hAFP (0.03 μg/ml to 10 μg/ml) were used for coating. Enzyme activity has been plotted as a function of concentration of the coating antigen at different antibody concentrations (Fig. 6A) and as a function of antiserum concentration at different concentrations of the coating antigen (Fig. 6B).

Figure 6A shows that any antigen concentration below 1 μg/ml, which gives a measurable response, can be chosen for coating. We will choose 0.1 μg of hAFP per milliliter for coating. We will then get the antibody concentration from Fig. 6B. This has to be chosen so that it is low enough

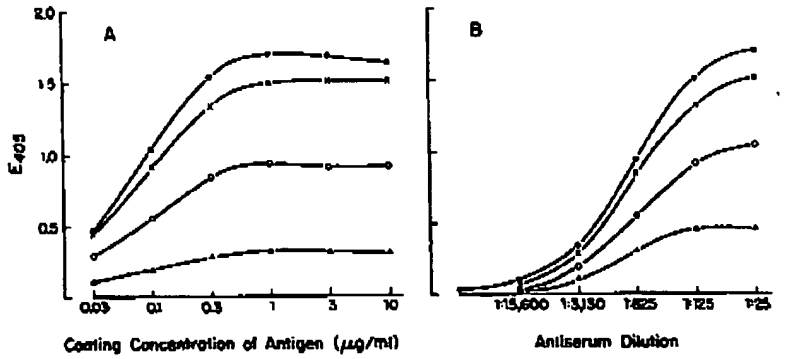

FIG. 6. Checkerboard titration of the antigen concentration used for coating and of antibody concentration. (A) Enzyme activity as a function of antigen concentration at coating for antiserum diluted 1:25 (●), 1:125 (×), 1:625 (○), and 1:3, 125 (△). (B) Enzyme activity as a function of antiserum dilution in wells coated with α-fetoprotein 1 μg/ml (●), 300 ng/ml (×), 100 ng/ml (○), and 30 ng/ml (△).

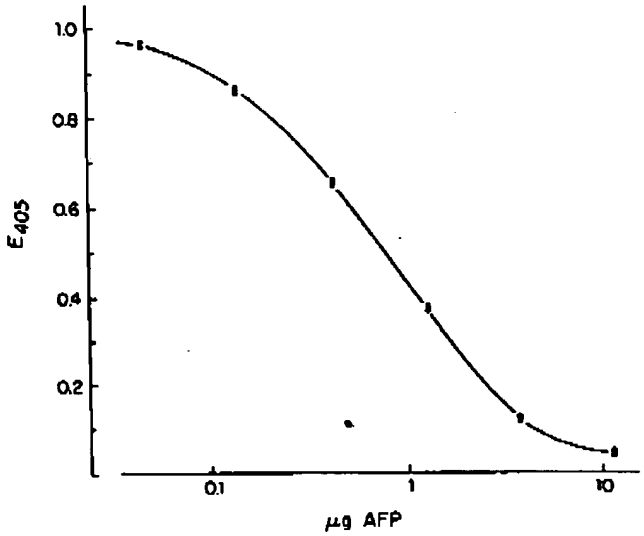

FIG. 7. Inhibition of binding of mouse anti-α-fetoprotein (AFP) to Microtiter wells coated with AFP by soluble AFP.

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to become a limiting factor in the assay. An antiserum dilution of 1:625 fulfills this requirement. The two-step competitive assay for hAFP is then performed in the following way.

Wells in a microtiter plate are coated with 0.1 μg of hAFP per milliliter in 0.1 *M* $NaHCO_3$. The plate is washed twice, and all wells are filled with 100 μl of incubation buffer. Fifty microliters of standard hAFP and unknown samples are added to a set of wells and then titrated by threefold dilutions. One hundred microliters of antiserum diluted 1:300 are then added to each well. The plate is incubated at room temperature for 3 hr. After another three washings, substrate is added and the enzyme is inactivated after 15 min. Figure 7 shows a standard curve for hAFP obtained in this assay. The inhibition curve has all the features of a regular standard curve in a competitive type assay.

Factors Involved in the Sensitivity of ELISA. As already mentioned, the experiments described in the previous paragraphs were not designed to obtain assays of highest possible sensitivity. The antiserum to HAFP we used was of relatively low affinity, we used short incubation times, and a short time for color development. To design an assay with optimal sensitivity, it is essential to (*a*) choose an antiserum of highest possible affinity; (*b*) use incubation times that allow an equilibrium between antigen and antibody; (*c*) use lower concentrations of antigen and antibody and longer times for color development.

With these factors taken into account, the sensitivity of an ELISA assay is comparable to that of radioimmunoassay.

Conclusion

Enzyme immunoassays are now firmly established as precise quantitative methods for the determination of various antigenic substances and antibodies. Proceedings of symposia,[30–32] reviews,[33–37] and monographs[38]

[30] Immunoenzymatic Techniques, INSERM Symposium No. 2 (G. Feldman, P. Druet, J. Bignon and S. Avrameas, eds.). North-Holland/Elsevier, Amsterdam, 1975.
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[32] "Enzyme Labeled Immunoassay of Hormones and Drugs" (S. B. Pal, ed.). De Gruyter, New York, 1978.
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[35] A. H. W. M. Schuurs and B. K. van Weemen, *Clin. Chim. Acta* 81, 1 (1977).
[36] E. Engvall, *Med. Biol.* 55, 193 (1977).
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[38] "Quantitative Enzyme Immunoassay" (E. Engvall and A. J. Pesce, eds.). *Scand. J. Immunol.* Suppl. 7, Vol. 8. Blackwell, Oxford, 1978.

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are available as sources of detailed descriptions of specific applications as well as general information on methodological aspects.

An extension of enzyme immunoassay is the enzyme affinity assay applicable to studies of nonimmunological interactions. This is already exemplified by the measurement of hormone using its receptor[39] and by our studies on the interaction of fibronectin with collagen.[10-12] Assays of these and similar principles might well become a new area of expression for EIA.

Acknowledgment

The preparation of this manuscript and parts of the original work described was supported by grants CA 16434, CA 19894, and CA 22108 from the National Cancer Institute, DHEW.

[39] F. S. Khan and B. B. Saxena, *in* "Enzyme Labeled Immunoassay of Hormones and Drugs" (S. B. Pal, ed.), p. 257. De Gruyter, New York, 1978.

[29] Electrode-Based Enzyme Immunoassays Using Urease Conjugates

By M. E. MEYERHOFF and G. A. RECHNITZ

The use of enzyme labels in place of radioisotopes for the measurement of antigens, antibodies, and haptens has stimulated the new and expanding field of enzyme immunoassay (EIA). This technique has been the focus of several recent reviews, [1-6] and its merits compared to radioimmunoassay (RIA) have been discussed.[7,8] In many cases, EIA can match RIA in terms of sensitivity and selectivity, yet has advantages of speed, convenience, and reduced cost. EIA sensitivity and simplicity is, however, dependent on the choice of enzyme label. It is the purpose of this work to introduce urease as a new enzyme label and to demonstrate the

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